Effects of Nebracetam on Synaptosomal Monoamine Uptake of Striatal and Hippocampal Regions in Rats

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Effects of nebracetam, a novel nootropic agent, on the synaptosomal uptake of neurotransmitter monoamines of the brain regions were examined. Striatal and hippocampal synaptosomes were isolated by the Percoll gradient method, and the striatal dopamine uptake and hippocampal serotonin uptake were measured in the presence of different concentrations (1 to 1000 μM) of nebracetam *in vitro*. A significant reduction in dopamine uptake in the striatum and serotonin uptake in the hippocampus was seen at concentrations of 100 μM or above. In *in vivo* microdialysis study, there were no appreciable changes in the extracellular concentrations of striatal dopamine and hippocampal serotonin when this agent at a dose of 30 mg/kg, which was effective in improving ischemic brain energy metabolism, was applied i.p. to the rat. The ineffectiveness of nebracetam in the *in vivo* microdialysis may be due to low levels of the concentration of nebracetam when the agent was administered i.p. at a dose of 30 mg/kg, since the brain blood concentration of this agent is pharmacokinetically estimated to be no more than 15 μM when this dose of nebracetam is employed. Thus, it is unlikely that this agent at a pharmacologically effective dose alters dopamine or serotonin uptake in the brain nerve terminal under normal conditions.

Key words dopamine; microdialysis; nebracetam; nootropic agent; serotonin; synaptosome

Nebracetam, 4-aminomethyl-1-benzyl-pyrrolidin-2-one hemifumarate, is a structural analogue of a pyrrolidine derivative aniracetam and has now been developed as a therapeutic drug on the sequelae of cerebral infarction and cerebral hemorrhage.¹⁾ This agent has cholinomimetic properties and cognitive enhancing actions, including enhancement of the firing rate of a single pyramidal cell from the hippocampal CA1,²⁾ an increase in the synthesis and release of acetylcholine (ACh) in the isolated ganglia of dogs,³⁾ the presence of binding affinity for muscarinic ACh receptor⁴⁾ and improvement of cognitive dysfunction induced by scopolamine.^{2,5)} Despite several observations on cholinomimetic action as above, little information is available concerning the effect of this agent on monoaminergic transmitters.

Aniracetam, a typical nootropic agent of a pyrrolidinone derivative, is known to affect monoaminergic neurotransmitters, including decreases in dopamine (DA) content of the striatum and hypothalamus and in serotonin (5-HT) content of the hypothalamus, and slowing of dopamine turnover in the striatum and 5-HT turnover in the hypothalamus.⁶⁾ Although administration of nebracetam did not affect the monoamine content of the rat brain *in vivo*,⁷⁾ its effect on monoamine uptake in nerve terminals has not been known. In the present study, we examined the effects of nebracetam on dopaminergic and serotonergic neurotransmitter uptake in brains. For this purpose, we determined the effects of this agent on DA and 5-HT uptake by the striatal and hippocampal synaptosomes *in vitro* and on extracellular concentrations of DA and 5-HT in the striatum and hippocampus, respectively, by an *in vivo* microdialysis method. The striatum and hippocampus were chosen because DA and 5-HT are located mainly in these brain regions.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 180—220 g, (Charles River Japan Inc., Atsugi) were used in the present study. The animals were maintained under artificial conditions at $23 \pm 1^\circ\text{C}$ with a constant humidity of $55 \pm 5\%$ with a cycle of 12 h light and 12 h dark, and had free access to food and tap water according to the Guideline of Experimental Animal Care issued by the Prime Minister's Office of Japan. This experimental protocol was approved by the University Committee of Animal Care and Welfare.

Preparation of Striatal and Hippocampal Synaptosomes Striatal and hippocampal synaptosomes were prepared by the method of Dunkley *et al.*⁸⁾ Briefly, 3 rats were anesthetized with diethylether and decapitated. Their brains were quickly isolated and the striatum and hippocampus of each hemisphere were dissected. The striatum and hippocampus were homogenized in ice-cold 0.32 M sucrose buffer containing 1 mM EDTA, pH 7.4, and 0.25 mM dithiothreitol in a Teflon-glass homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C . The supernatant fluid was subsequently centrifuged at $32500 \times g$ for 5 min at 4°C on a discontinued 4-layer Percoll gradient (23, 15, 10 and 3% of Percoll). The interface fraction between 23 and 15% Percoll layers was collected and washed twice with the reaction buffer containing the following composition (mM): NaCl 118.1, KCl 4.7, CaCl_2 11.2, MgCl_2 1.2, NaHCO_3 24.9, glucose 10.0, pargyline 0.15, by centrifugation at $15000 \times g$ for 15 min at 4°C . The pellet was resuspended in the reaction buffer as above and used for the monoamine uptake assay. The protein determination was carried out by the method of Lowry *et al.*⁹⁾

DA and 5-HT Uptake Determination of DA and 5-HT uptake was performed according to the method described previously.¹⁰⁾ The isolated synaptosomes were suspended

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in the reaction buffer as above and preincubated for 5 min at 37 °C. The uptake was started by the addition of ^3H -dopamine or ^3H -5-hydroxytryptamine in 1 ml of the reaction mixture containing either the striatal or hippocampal synaptosomes, respectively, at a final concentration of 0.1 μM . The incubation was stopped at 5 min for DA or 10 min for 5-HT after the incubation by the addition of 4 ml of ice-cold buffer. The filter was placed in scintillation vials and their radioactivity was determined by a liquid scintillation counter (LSC-3500, Aloka, Tokyo). Values were corrected for the respective accumulation of radioactivity in the synaptosomes incubated at 0 °C. The activity was expressed as pmol/mg protein.

Determination of Extracellular DA and 5-HT Concentrations *in Vivo* Determination of extracellular DA and 5-HT concentrations was performed by the intracerebral microdialysis method as described elsewhere.¹¹⁾ Rats were anesthetized with 400 mg/kg chloral hydrate i.p., then placed on a stereotactic frame for microdialysis probe implantation. The right part of the skull was exposed and a 1-mm hole was drilled on the skull with a minidrill (No. 28400, Proxxon, Germany). A guide cannula for microdialysis probe was inserted into the right striatum or hippocampus at the following coordinates: 0.2 mm anterior, 3.0 mm lateral to bregma and 3.5 mm below dura for the striatum, and 7.5 mm posterior, 5.0 mm lateral to bregma and 2.5 mm below dura for the hippocampus, according to the Atlas of Paxinos and Watson.¹²⁾ After implantation of the guide cannula, a microdialysis probe with a 3 mm long membrane (Eicom, Kyoto) was inserted into the striatum or hippocampus through the guide cannula. The microdialysis probe was perfused with Ringer's solution at a flow rate of 2 $\mu\text{l}/\text{min}$ with a micro-infusion pump (EP-60, Eicom, Kyoto). After a 2-h stabilization, the microdialysis perfusate was injected to HPLC-ECD by an autoinjector (AS-10, Eicom, Kyoto) at 20 min intervals. High K^+ -stimulation was performed by the perfusion of 100 mM KCl for 20 min. The high K^+ solution had the same composition as that of the Ringer's solution except that NaCl was replaced with KCl to provide 100 mM KCl. To determine the extracellular DA and 5-HT concentrations, the microdialysis perfusate in the chromatographic column (Eicompak CA-5ODS, Kyoto) was eluted at 25 °C with a solution containing 0.1 M Na_2HPO_4 , 0.1 M NaH_2PO_4 , 20% methanol, 500 mg/l sodium 1-octanesulfonate and 50 mg/l EDTA- Na_2 at a flow rate of 0.22 ml/min (EP-300, Eicom, Kyoto). The eluate was detected at 500 mV by an electrochemical detector (ECD-300, Eicom, Kyoto).

In a previous study,¹³⁾ we examined the effects of different concentrations of nebracetam ranging from 3 to 100 mg/kg on cerebral energy metabolites of the microsphere embolism-induced cerebral ischemia in *in vivo* rats. It was found that 30 mg/kg nebracetam most effectively improved the ischemia-induced damage to energy metabolites of the brain regions. Thus, we used 30 mg/kg of nebracetam as a pharmacologically effective dose in the *in vivo* study.

Statistics The results are expressed as the mean $\pm$ S.E.M. Statistical significance for comparison of synaptosomal uptake was evaluated by analysis of variance

(ANOVA) followed by Bonferroni's multiple comparison, whereas that for comparison of monoamine release was by two-way repeated-measures ANOVA. Differences with a probability of 5% or less were considered to be statistically significant ($p < 0.05$).

RESULTS

Synaptosomal Uptake of Monoamines The direct effects of nebracetam on the striatal and hippocampal synaptosomal uptake of DA and 5-HT, respectively, were determined under *in vitro* conditions. As shown in Fig. 1, the synaptosomal uptake of DA in the striatum was significantly decreased at concentrations of 100 μM or above of nebracetam. Similarly, the synaptosomal uptake of 5-HT in the hippocampus was significantly decreased at concentrations of 100 μM or above of nebracetam (Fig. 2).

Changes in Extracellular Concentrations of DA in the Striatum and 5-HT in the Hippocampus To examine the effect of nebracetam on DA release in the striatum and 5-HT release in the hippocampus, microdialysis was per-

Fig. 1. Effects of Different Concentrations (10 to 1000 μM) of Nebracetam on Dopamine (DA) Uptake of the Striatal Synaptosomes *in Vitro*

Each bar represents the mean $\pm$ S.E.M. of 6 experiments. * Significantly different from control value (C) ($p < 0.05$).

Fig. 2. Effects of Different Concentrations (10 to 1000 μM) of Nebracetam on Serotonin (5-HT) Uptake of the Hippocampal Synaptosomes *in Vitro*

Each bar represents the mean $\pm$ S.E.M. of 6 experiments. * Significantly different from control value (C) ($p < 0.05$).

Fig. 3. Extracellular Concentration of Dopamine (DA) in the Striatal Region of Normal Rats with (Closed Circles) and without (Open Circles) 30 mg/kg Nebracetam-Treatment i.p. as Indicated by Arrows

High potassium-induced release of dopamine was performed by the perfusion of 100 mM KCl for 20 min as indicated by a solid bar. Each point represents the mean $\pm$ S.E.M. of 4 experiments.

Fig. 4. Extracellular Concentration of Serotonin (5-HT) in the Hippocampal Region of Normal Rats with (Closed Circles) and without (Open Circles) 30 mg/kg Nebracetam-Treatment i.p. as Indicated by Arrows

High potassium-induced release of serotonin was performed by the perfusion of 100 mM KCl for 20 min as indicated by a solid bar. Each point represents the mean $\pm$ S.E.M. of 4 experiments.

formed in the striatal and hippocampal regions, respectively, of freely moving rats *in vivo*. The administration of nebracetam did not alter the basal levels of the striatal DA (36.45 ± 3.54 pg/40 μ l for control and 28.05 ± 4.08 pg/40 μ l for nebracetam-administered rat) or hippocampal 5-HT (1.14 ± 0.40 pg/40 μ l for control and 0.97 ± 0.11 pg/40 μ l for nebracetam-administered rat). The concentrations of both monoamines in each brain region were greatly increased after the administration of 100 mM potassium chloride (Figs. 3 and 4). A high K^+ -induced increase in extracellular concentrations of DA tended to be reduced by pretreatment with 30 mg/kg nebracetam i.p., but it was not significant (Fig. 3). This pretreatment did not affect the high K^+ -induced increase in the extracellular con-

centration of 5-HT in the hippocampus either (Fig. 4). In a preliminary study, we used various doses of nebracetam ranging from 10 and 30 mg/kg *p.o.* and *i.p.* to test their effects on the uptake and/or release of monoamines, and found that there were no significant effects of nebracetam on the extracellular concentrations of DA or 5-HT in the brain regions.

DISCUSSION

In the present study we examined the *in vitro* effects of nebracetam on DA or 5-HT uptake in the striatal or hippocampal synaptosomes, respectively. A significant inhibition of DA and 5-HT uptake by the striatal and

hippocampal synaptosomes was seen at a concentration of 100 μ M or above of nebracetam. This implies that nebracetam is capable of inhibiting neurotransmitter uptake in the striatum and hippocampus, and thus capable of augmenting DA-mediated stimulation in the striatum or 5-HT-mediated stimulation in the hippocampus.

To ascertain this possibility, we determined changes in the extracellular DA or 5-HT concentration in the striatal or hippocampal region, respectively, of nebracetam-administered rats *in vivo*, since the extracellular concentration of DA or 5-HT would be increased after inhibition of the synaptosomal uptake of DA or 5-HT. Unfortunately, no appreciable effects of nebracetam on changes in extracellular concentration of DA or 5-HT were detected under *in vivo* conditions. As described in the "Methods" section, the dose employed in the present study (30 mg/kg) was chosen because it improved the impaired brain energy metabolism of microsphere-embolized rats *in vivo*.¹³⁾ It is reported that nebracetam at doses ranging from 10 to 32 mg/kg caused a dose-related reduction in the increase in errors expected in scopolamine-treated rats.⁵⁾ Furthermore, we observed in a preliminary study that a monoamine transporter inhibitor, desipramine, when administered i.p. at a dose of 7.25 mg/kg under the same experimental conditions as those in the present study, markedly increased the high potassium-induced increase in the extracellular DA concentration in the striatum (about a 5-fold increase). Thus, the results of the *in vivo* microdialysis study suggest that no appreciable changes in the uptake of DA or 5-HT would be caused by administration of the pharmacological dose of nebracetam.

It is possible that extracellular concentrations of DA and 5-HT are appreciably influenced by alterations in the synaptosomal synthesis and catabolism of DA and 5-HT caused by an agent. We observed that there were no appreciable effects of the repeated treatment with 30 mg/kg/d nebracetam on DA or 5-HT, and their metabolites of the brain regions in intact animals (unpublished observations). Hashimoto *et al.*⁷⁾ also reported no changes in DA or 5-HT and their metabolites of the normal rat brain after a single administration of 100 mg/kg nebracetam. Thus, it is unlikely that DA and 5-HT synthesis and catabolism are influenced by nebracetam under the present experimental conditions. The results of the *in vivo* microdialysis appear to disagree with those of the *in vitro* study on synaptosomal monoamine uptake.

Momose *et al.*¹⁴⁾ have shown the pharmacokinetic profile of nebracetam administered orally or intravenously into the rat. According to their results, this drug was distributed in the blood with a maximal concentration of 19.5 μ g/ml 24 min after the oral administration of 5, 50 or 100 mg/kg nebracetam in rats, and the concentration declined soon after it reached maximal concentration. Since the ratio of protein binding of this drug was 30.2% in rats 30 min after the administration, the free form of this drug in the blood would be 8.2 μ g/ml (28 μ M) or less when 30 mg/kg of nebracetam, a dose that we used, is orally or intraperitoneally administered. It is also reported

that the ratio of brain concentration to blood concentration is 0.557.¹⁴⁾ Although a substantial concentration of this drug in the brain blood under the present experimental conditions is unknown, the brain blood concentration would be no more than 15 μ M when 30 mg/kg nebracetam is orally or intraperitoneally administered into the rat, as calculated on the basis of the pharmacokinetic data as above. It should be noted that our results on the DA and 5-HT uptake of the striatal and hippocampal synaptosomes appeared not to be affected by this concentration of nebracetam. The ineffectiveness of nebracetam in the extracellular concentration of monoamines may be attributed to low levels of the substantial concentration of this agent in the brain regions of the nebracetam-administered animals. Thus, it is unlikely that this agent at its pharmacological dose would inhibit the uptake of dopamine and 5-HT in the brain nerve terminal under normal conditions.

As described in our Introduction, it has been reported that nebracetam did not affect dopamine or serotonin content in brain regions under normal conditions.⁷⁾ Furthermore, Iwasaki *et al.*¹⁵⁾ have shown that nebracetam did not affect brain norepinephrine levels in normal rats, although it antagonized scopolamine-induced decrease in tissue norepinephrine levels. These observations by others and our results in the present study suggest that this agent at a pharmacologically effective dose appears not to exert any effect on monoamine uptake in brains, at least under normal conditions.

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